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Mitochondrial GCD1 Dysfunction Reveals Reciprocal Cell-to-Cell Signaling during the Maturation of *Arabidopsis* Female Gametes

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SUMMARY

Cell-to-cell communication in embryo sacs is thought to regulate the development of female gametes in flowering plants, but the details remain poorly understood. Here, we report a mitochondrial protein, GAMETE CELL DEFECTIVE 1 (GCD1), enriched in gametophytes that is essential for final maturation of female gametes. Using *Arabidopsis gcd1* mutants, we found that final maturation of the egg and central cells is not required for double fertilization but is necessary for embryogenesis initiation and endosperm development. Furthermore, nonautonomous effects, observed when GCD1 or AAC2 function is disrupted, suggest that mitochondrial function influences reciprocal signaling between central and egg cells to regulate maturation of the partner (egg or central) cell. Our findings confirm that cell-to-cell communication is important in functional maturation of female gametic cells and suggest that both egg and central cells sense and transmit their mitochondrial metabolic status as an important cue that regulates the coordination of gamete maturation.

INTRODUCTION

Well-developed egg and central cells, which act as female gametes, are prerequisites for double fertilization in angiosperms (Ma and Sundaresan, 2010; Yadegari and Drews, 2004). During double fertilization, the two female gametic cells fuse with two sperm cells, producing an embryo and endosperm. Successful interaction with the two sperm cells during double fertilization requires simultaneous maturation of the egg and central cells. The mechanism controlling the coordinated development of the female gamete cells remains unknown but is thought to involve cell-to-cell communication, which usually involves signal transduction among neighboring cells via a physical connection or secreted extracellular cue.

Early in female gametophyte development, multiple plasmodesmata link the functional megaspore and the surrounding nucellar cells, which are thought to be important for the differentiation of female gametophytes (Bajon et al., 1999). Genetic analysis has suggested that cross-communication occurs between the female gametophyte and sporophytic tissues in the ovule, highlighting the important role of the chalaza and integument in mediating the exchange of information necessary for development of the embryo sac (Bencivenga et al., 2011). Experiments in which fluorescent morpholino antisense oligomers (Okuda et al., 2009) and small fluorescent tracers were injected into the cytoplasm of *Torenia fournieri* central cells have indicated the presence of a symplastic connection between the egg cell, synergids, and central cell (Han et al., 2000), implying molecular transportation among these cells. However, when the 27 kDa green fluorescent protein (GFP) molecule (Liarzi and Epel, 2005) is expressed in *Arabidopsis*, no diffusion of the fluorescent signal from one cell to the other in the embryo sac is observed (Punwani et al., 2007; Steffen et al., 2007). Thus, additional investigations are required to confirm that communication between the gametic cells occurs via physical connections.

Communication between the central and antipodal cells was recently reported by Kägi et al. (2010), who examined central cells defective for *FIONA*, a gene normally expressed in the central cell but not in antipodals. *FIONA* is required for the final maturation of the central cell. The *fiona* defect disrupted the timing of antipodal cell degradation, providing convincing evidence that the central cell determines the life span of antipodal cells. It has been also shown in maize that *ZmEAL1* encoding a secreted, non-cell-autonomous peptide specifically expresses in the egg cell and functions in preventing antipodal cells from adopting central cell fate (Krohn et al., 2012). Other researchers have suggested that central cell–egg cell communication is involved in cell fate patterning during embryo sac development (Chevalier et al., 2011), thereby influencing female gametic development and fate determination. Clearly, this fascinating hypothesis warrants further investigation.

As the central and egg cells reach maturation, they become functionally specialized such that their fates diverge after fertilization. However, the mechanism that regulates the final maturation and functional specification of female gametes remains poorly understood. In animals, active transcription occurs as

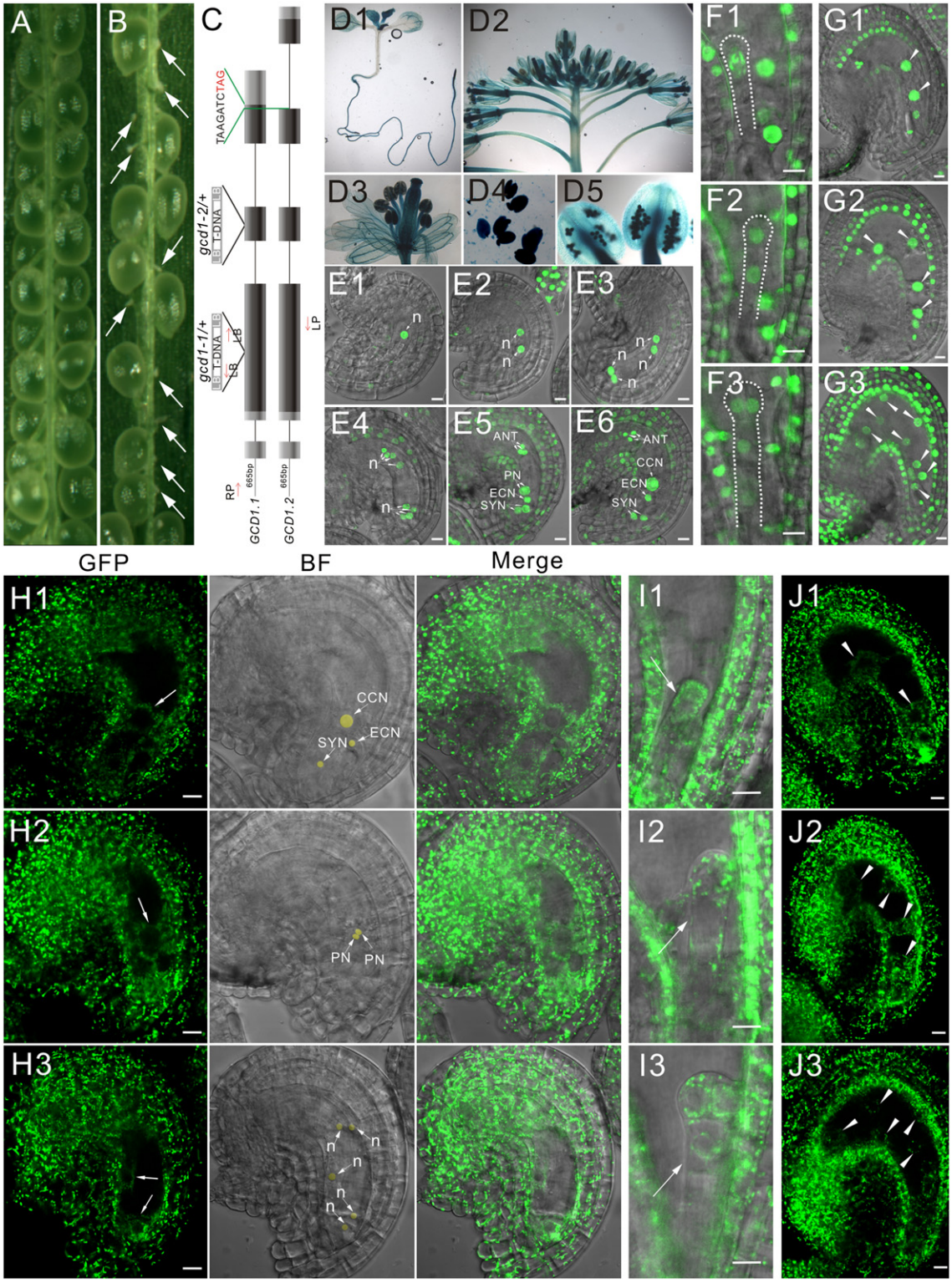


Figure 1. *gcd1/GCD1* T-DNA Insertion and GCD1 Expression Pattern Analysis

(A) Wild-type silique showing full seed set.

(B) Silique of a selfed *gcd1/GCD1* plant containing normal and aborted (arrows) seeds.

(C) Diagram of the genomic locus of *GCD1*. The two T-DNA insertions disrupt the first and the second exon in the *gcd1-1* and *gcd1-2* allele, respectively. The change of DNA sequence between two transcripts in *GCD1* is indicated. Exons are depicted in bars; introns are depicted in lines. UTRs are indicated in light gray; coding exons are indicated in dark gray. The dashed lines indicate upstream sequence of *GCD1*.

early as the primary oocyte stage, and the mRNAs and proteins necessary for fertilization and early embryogenesis have been synthesized by the time the oocyte progresses to metaphase II during oogenesis. As a result, embryogenesis initiation and early development in animals relies primarily on materials stored in the oocyte prior to fertilization (Schultz, 2005; Stitzel and Seydoux, 2007). However, it is still not clear when the molecular set up necessary for fertilization and embryo initiation is established in flowering plants. Only two stages of egg cell development, the immature and mature stages, have been proposed for maize (Mól et al., 1994; Van Lammeren, 1986). Pollination can accelerate egg cell maturation (Mól et al., 2000), and in maize and soybean, the egg cytoplasm becomes less dense during egg cell maturation (Folsom and Cass, 1990; Mól et al., 2000). During maturation of the *Pelargonium zonale* egg cell, replication of the mitochondrial DNA has been reported to gradually increase, with the small ring- or string-like mitochondria becoming giant, cup-shaped mitochondrial complexes (Kuroiwa et al., 1996). These observations suggest that the plant egg cell may require a maturation or specification stage to be functional, but the biological impact of this putative process on fertilization and embryogenesis is unknown.

In the present report, we describe our functional gene analysis showing that the *Arabidopsis* gene *GAMETE CELLS DEFECTIVE1* (*GCD1*) is essential for female gametic cell maturation and that the maturation process is necessary for triggering *Arabidopsis* embryogenesis and endosperm development. We also provide strong evidence of cell-to-cell communication between central and egg cells. The two types of gametic cells send developmental signals to one another, and these signals are required for the final maturation of both cells. Our results confirm the critical role of female gametic maturation in embryogenesis and endosperm development. The presence of this reciprocal signal from central and egg cells provides a mechanistic basis for the coordinated maturation of female gametic cells.

RESULTS

GCD1 Encodes a Conserved Protein

From our *Arabidopsis* mutant library, we obtained one mutant, *gcd1-1/GCD1*, with a single T-DNA insertion. All of the mutant plants were heterozygous, and 48% of the seeds (n = 1,698) were aborted (Figures 1A and 1B). In reciprocal crosses between the mutant heterozygous for the T-DNA insertion and the wild-type plant, the mutation dramatically impaired female gametophyte (FG) functioning and completely disrupted male gametophyte functioning (Table S1available online). Another T-DNA insertion mutant of the same allele (Figure 1C), *gcd1-2/GCD1*, exhibited similar defects in male and female gametophyte function (Table S1).

We identified the T-DNA insertion in *GCD1* (Figure 1C). A rescue construct introduced into heterozygous *gcd1/+* mutants was able to revert the mutant phenotypes (Table S2; Figure S1A). Plants homozygous for the *gcd1* allele (Figures S1B–S1G) and rescued constructs were obtained and showed full seed sets of siliques, indicating complete complementation. These data confirmed that the mutant phenotype was indeed caused by the loss of function of *GCD1*.

GCD1 Is Expressed at High Levels in Reproductive Organs of *Arabidopsis*

The results of *pGCD1::GUS* signal analysis in transgenic *Arabidopsis* plants suggested universal expression of the *GCD1* gene with preferential expression in reproductive organs (Figure 1D). *pGCD1::H2B_GFP* was also detected in the female gametophytes at different stages, as well as in early embryos and endosperms (Figures 1E–1G).

We next generated a fusion protein construct, GCD1-GFP, comprising the *GCD1* promoter and the entire GCD1 coding region fused to the GFP coding sequence. This construct was sufficient to rescue the mutant phenotype in the *gcd1/+* background (Table S2), suggesting that this construct plays a role similar to that of endogenous GCD1. Transgenic plants of the wild-type background were then analyzed. At stage FG7-FG5, GCD1-GFP expression was easily detectable in mature female gametophytes (Figure 1H), early embryos (Figure 1I), and endosperms (Figure 1J), implying that GCD1 has an important function during these periods.

GCD1 Is Required for Pollen Tube Growth and Style Penetration

We first analyzed the role of *GCD1* in male gametophyte development. The *gcd1* pollen was labeled with GFP to facilitate observation (Figure 2A). Staining of the DNA with 4′-6-diamidino-2-phenylindole indicated no notable effects of the mutation on sperm and vegetative cell formation (Figures 2B and 2C). All *gcd1/GCD1* pollen germinated well in vitro, but pollen tubes with GFP signals (average length = 124 μm, n = 230) were markedly shorter than those without GFP signals (average length = 230 μm, n = 136), indicating abnormal growth of the *gcd1* pollen tube (Figures 2D–2F). Pollination of wild-type pistils with *gcd1/GCD1* pollen in an in vitro–in vivo assay revealed that the *gcd1*

(D) Signal distribution of *pGCD1::GUS*. (D1) GUS activity in the leaf, root. (D2) *pGCD1::GUS* broadly expressed in the inflorescence. (D3) GUS activity in the flower at stage 12, mainly in the stamen and pistil. (D4 and D5) Strong GUS activity in ovules (D4) and in pollen (D5).
(E–G) *pGCD1::H2B_EGFP* expression. (E) *pGCD1::H2B_EGFP* expressed in the different stage of FG: FG1 (E1), FG2 (E2), FG4 (E3), FG5 (E4), FG6 (E5), and FG7 (E6). (F) *pGCD1::H2B_EGFP* expressed in the zygote (F1), two-celled proembryo with an apical and a basal cell (F2), proembryo with divided apical cell (F3). The dashed lines indicate the edges of the embryos. (G) *pGCD1::H2B_EGFP* expressed in the early endosperm at two endosperm nucleus stage (G1), four endosperm nucleus stage (G2), and eight endosperm nucleus stage (G3). The arrowheads indicate the endosperm nuclei with GFP signal.
(H–J) *pGCD1::GCD1_EGFP* Expression. (H1) GCD1 protein expressed in female gametophyte at the stage FG7. (H2) Before polar nucleus fusion (stage FG6). (H3) Before cellularization of embryo sac (stage FG5). The arrows indicate GFP signals in the FG. (I1) GCD1 expressed in the zygote. (I2) GCD1 expressed in a two-celled proembryo with an apical and a basal cell. (I3) GCD1 expressed in the proembryo with divided apical cell. The arrows indicate zygote or early proembryo. (J) GCD1 expressed in the early endosperm at two endosperm nucleus stage (J1), four endosperm nucleus stage (J2), and eight endosperm nucleus stage (J3). The arrowheads indicate the cytoplasmic domains with GFP signal around each of endosperm nuclei. CCN, central cell nuclei; ECN, egg cell nucleus; SYN, synergid cell nucleus; ATN, antipodal cell nuclei; PN, polar nuclei; n, nucleus. Bars = 10 μm.
See also Figure S1 and Tables S1 and S2.

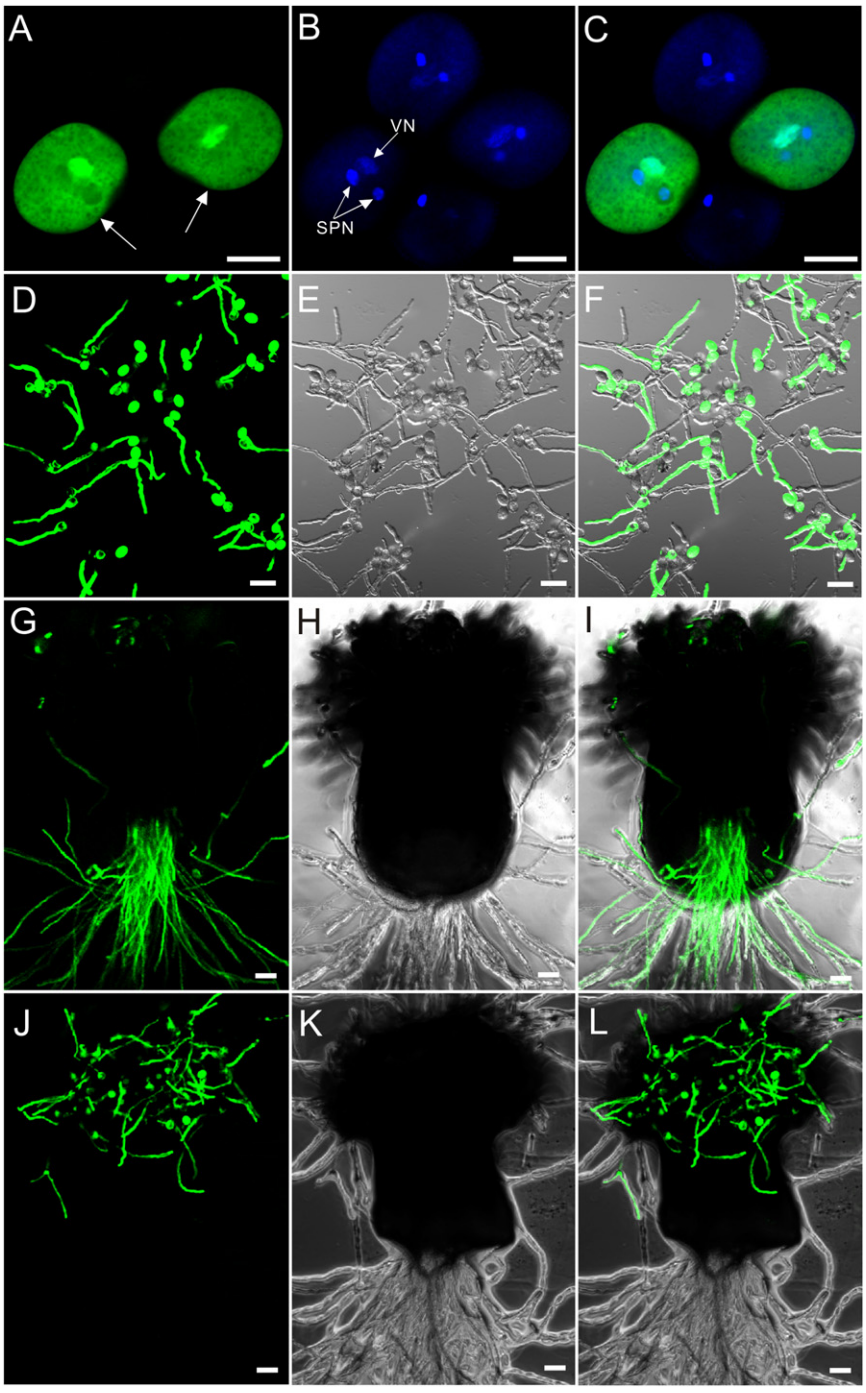


Figure 2. *gcd1* Mutation Affected Pollen Germination and Penetration into the Style

(A–C) DAP1 staining of a *gcd1/GCD1* pollen tetrad. (A) *pLAT52::EGFP*; (B) DAP1; (C) merged image of (A) and (B). Arrows indicate the pollen with GFP fluorescence. VN, vegetative cell nucleus; SPN, sperm cell nuclei. Scale bars, 10 μm.

(D–F) *gcd1/GCD1* pollen germination in vitro. (D) *pLAT52::EGFP*; (E) bright field; (F) merged image.

(G–L) In vivo-in vitro assay after the wild-type pistils pollinated with pollens carrying *pLAT52::EGFP* (G–I) and with *gcd1/GCD1* pollens (J–L). (G and J) *pLAT52::EGFP*; (H and K) bright field; (I and L) merged images. Scale bars, 50 μm.

pollen could germinate on the stigma but could not penetrate into the style (Figures 2G–2L).

GCD1 Is Required for Female Gametic Cell Maturation

Phenotype analysis of *gcd1*/+ mutants 12 and 72 hr after emasculation showed that 37% (n = 359) and 30% (n = 374), respectively, of the embryo sacs contained unfused polar nuclei (Figures 3A–3C), indicating a block to polar nuclei fusion in the *gcd1* female gametophyte and an important role for *GCD1* in the central cell maturation.

A recent report revealed that a developmental defect of central cells, indicated by unfused polar nuclei, affects antipodal degeneration (Kägi et al., 2010). As expected, we also observed persistent antipodal cells in *gcd1*/+ mutant embryo sacs (Figures 3B and 3D), providing further evidence of the abnormality of the central cells in *gcd1*/+ mutants.

Next, we observed the morphology of *gcd1* egg cells. By careful measurement and calculation of egg cell size and length (Figures 3M and 3N), we determined that the mutant egg cells were significantly smaller and shorter than wild-type egg cells (Figures 3O and 3P). As shown in Figures 3Q and 3R, a comparison of the coefficient of variance (CV) of egg cell development suggested that the wild-type egg cells developed much more uniformly than did *gcd1*/+ egg cells, as the CV of the latter was much greater, suggesting variable egg cell development. Only 50% of the *gcd1*/+ egg cells were the size of normal mature eggs (200 μm^2), whereas 90% of the wild-type egg cells were the size of normal mature eggs (Figures 3Q and 3R). Furthermore, the density distribution curves of wild-type and *gcd1/GCD1* egg cell size were significantly different (Figures S2A and S2B). All of these results indicate that egg cell development is affected in *gcd1*/+ mutants.

When we prepared a crossed line, *DD45/DD45 gcd1*/+, in which the egg cell was labeled with GFP (Figures S2C and S2D), we found smaller egg cells in the *gcd1* embryo sacs (Figure S2E). This result also suggests that *GCD1* is required for the final development of egg cells.

The defects seen in *lachesis* and *clotho* mutants are likely to be a consequence of a misspecified central cell or egg cell (Gross-Hardt et al., 2007; Moll et al., 2008). When we introduced two central-cell markers and one egg-cell marker into *gcd1*/+ mutant and wild-type plants, all markers (Portereiko et al., 2006a;

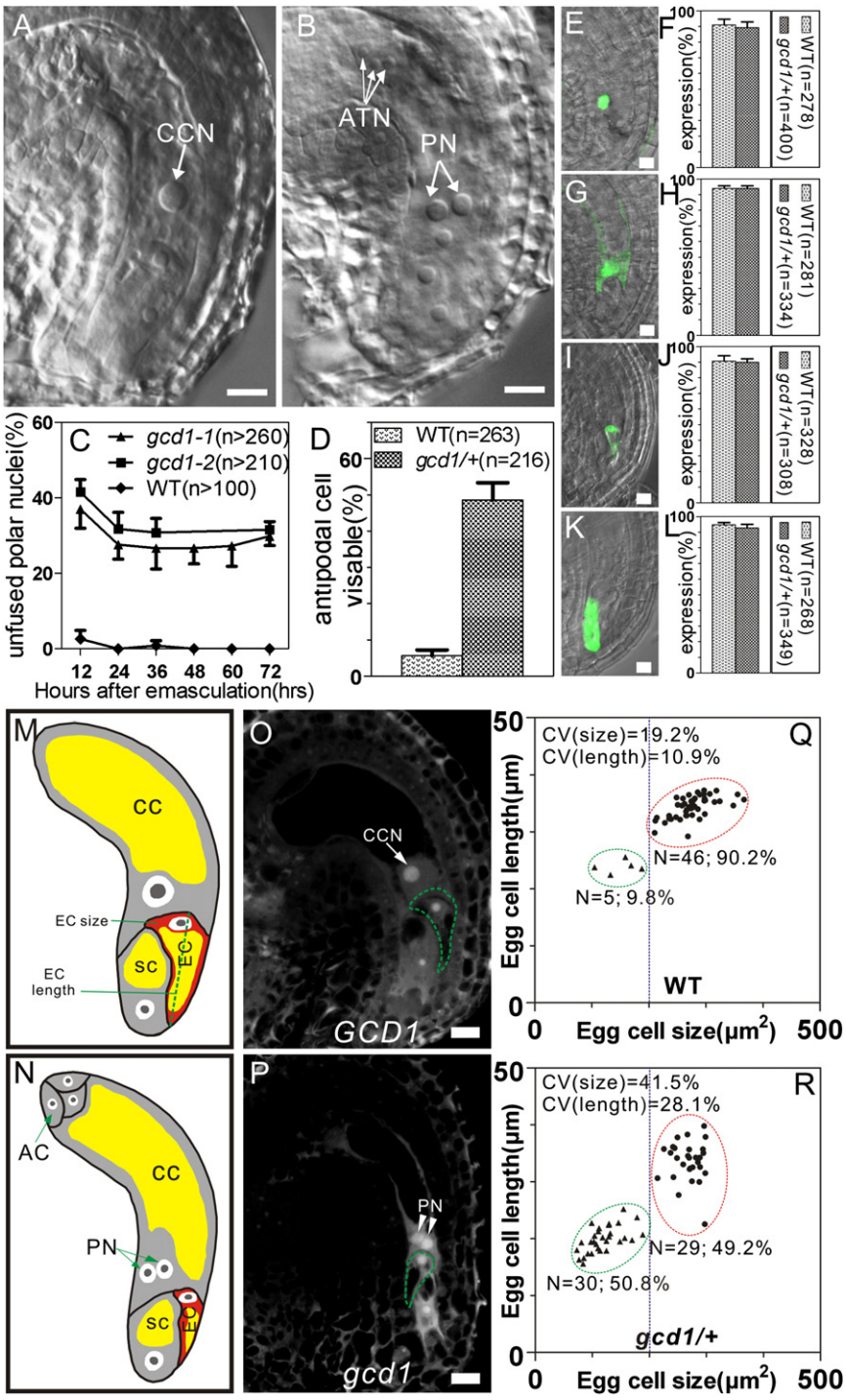


Figure 3. *gcd1*/+ Exhibits the Defects in Female Gamete Maturation

(A) Wild-type gametophyte containing a secondary nucleus in the central cell.

(B) *gcd1* gametophyte containing unfused polar nuclei and persisting antipodal cell.

(C) Frequencies of unfused polar nuclei at different developmental stages.

(D) Frequencies of persistent antipodal cells. Gametophytes were analyzed 2 days after emasculation (DAE).

(E–L) Expression of cell-type-specific marker genes in the *gcd1*/+ mutant. (E) Expression of the central cell marker *pDD65::H2B_EGFP* in the polar nuclei. (F) Frequencies of ovules expressing *pDD65::H2B_EGFP*. (G) Expression of the central cell marker *AGL80-EGFP* in the immature central cell. (H) Frequencies of ovules expressing *AGL80-EGFP*. (I) Expression of the egg cell marker *DD45-GFP* in the immature egg cell. (J) Frequencies of ovules expressing *DD45-GFP*. (K) Expression of the synergid cell marker *DD31-GFP*. (L) Frequencies of ovules expressing *DD31-EGFP*.

(M and N) Depictions of the female gametophyte in the *GCD1* (M) and *gcd1* (N) at stage FG7.

(O and P) CLSM analysis of wild-type (O) and *gcd1* (P) female gametophyte. The dashed line marks the egg cell.

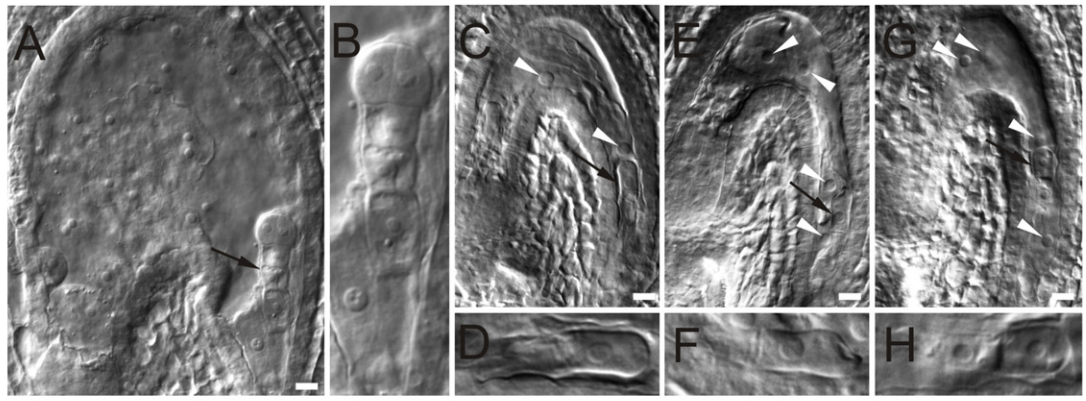
(Q and R) The distribution of egg cell sizes and lengths in the wild-type (Q) and *gcd1*/+ (R) female gametophyte. The dash line at 200 μm^2 was defined to estimate normal egg cells (red ellipse) and immature egg cells (green ellipse). All the egg cells used for measurement were fixed unless specially noted. CC, central cell; EC, egg cell; SC, synergid cell; AC, antipodal cell; PN, polar nuclei; CCN, central cell nuclei; ATN, antipodal nuclei. CV, coefficient of variance. Scale bars, 10 μm. Error bars indicate SD.

See also Figure S2.

Steffen et al., 2007) were expressed at comparable levels in the two types of plants (Figures 3E–3J), suggesting that cell identity was maintained in the mutants, but late maturation of the female gamete was interrupted. The overall morphology of synergids in *gcd1*/+ mutants was also normal, as was the expression of a synergid-cell-specific marker (Steffen et al., 2007) (Figures 3K and 3L).

Mutant *gcd1* Central and Egg Cells Are Capable of Fertilization but Not Embryogenesis Initiation and Endosperm Proliferation

When we used a *pLAT52::GUS* plant (Twell et al., 1989) as a pollen donor, pollen tube guidance in the immature *gcd1* female gametes was not impaired (data not shown). We then analyzed double fertilization in *gcd1*/+ mutant ovules pollinated with wild-type pollen. Two days after pollination (DAP) of the *gcd1*/+ heterozygous pistils, 87.1% of early seeds (n = 327) contained both an embryo and endosperm, indicating fertilization of the defective gametic cells (Figure 4I). However, 39.5% of the seeds subsequently aborted, a result clearly different from that seen with wild-type seeds (Figures 4A and 4B). Almost all of the zygotes in the aberrant early seeds arrested immediately (Figures 4C–4F), but 4.3% of them divided once before arrest (Figures 4G–4I). The early endosperm arrested in the two- or four-nucleus



female× male	Endosperm (%)			Embryo (%)			unfertilized	n
	2nuc	4nuc	>4nuc	zygote	Two cells	>two cells		
GCD1/gcd1-1×WT	23.2%	16.3%	47.6%	35.2%	4.3%	47.6%	12.9%	327
GCD1/gcd1-2×WT	27.6%	14.5%	51.1%	41.2%	0.9%	51.1%	6.8%	249
WT×WT	0.0%	0.0%	90.1%	0.0%	1.3%	88.8%	9.9%	224

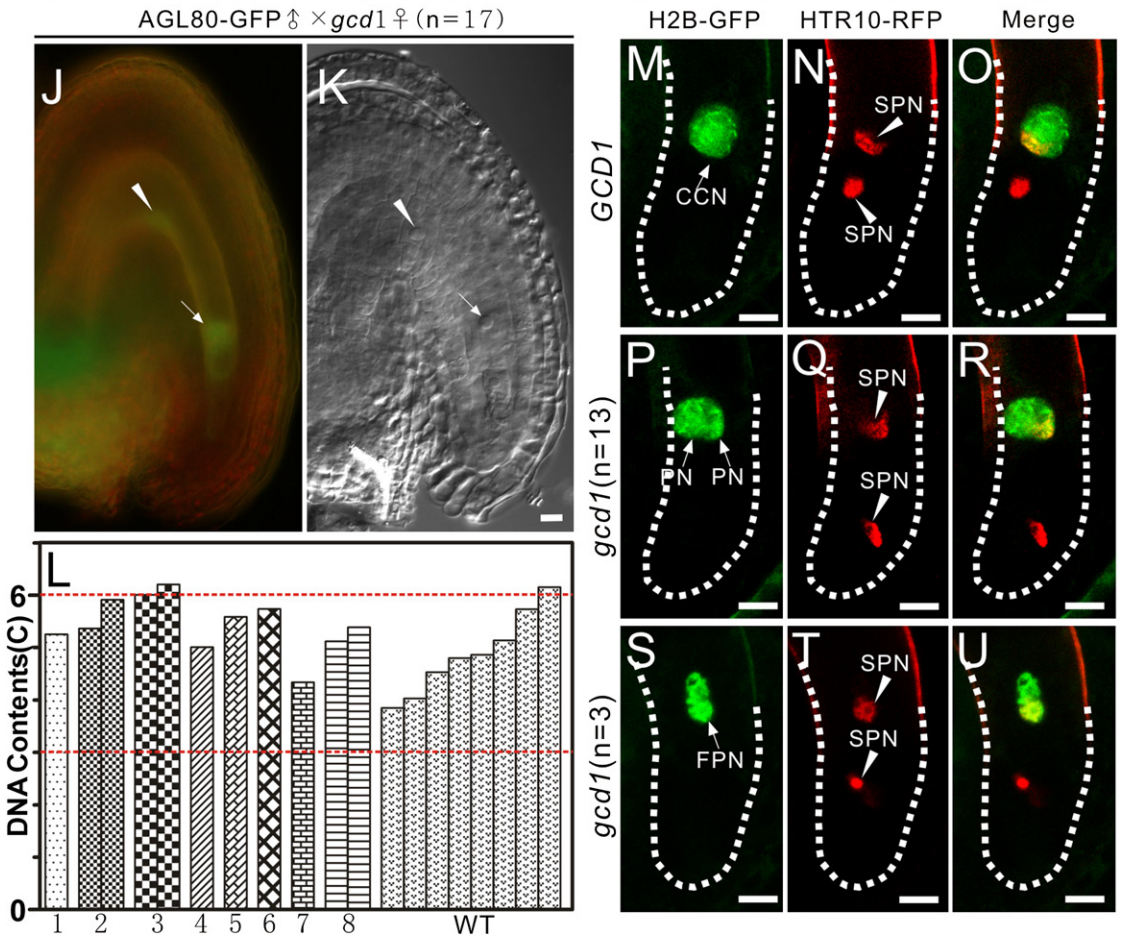


Figure 4. *gcd1*-Defective Female Gametes Are Capable of Fusion with Sperm cells, but the Development of Zygote and Early Endosperm Is Arrested

(A–H) Embryo and endosperm development in *GCD1* female gametophytes (A) and in *gcd1* female gametophytes (C, E, and G) 2 days after pollination with wild-type pollen. (B) Magnification of embryo in (A). (D) Magnification of zygote in (C). (F) Magnification of zygote in (E). (H) Magnification of two-celled proembryo in (G). Arrows indicate the developing global embryo and arrested zygote or two-celled proembryo. Arrowheads indicate the arrested early endosperm nuclei.
(I) Frequencies of different arrested embryo and endosperm when *gcd1*/+ and wild-type pistils pollinated with wild-type pollen at 2 DAP. nuc, nucleate.

stage (Figures 4C, 4E, and 4G). Observations of pistils at 3 DAP revealed that the aborted seeds had begun to collapse. Taken together, these results indicate blockage of embryogenesis initiation and early endosperm proliferation, with no opportunity for further development, in *gcd1*/+ seeds.

The paternal expression pattern of the GRP23-GUS (Ding et al., 2006; Tsukamoto et al., 2010) and AGL80-GFP (Portereiko et al., 2006a) markers in *gcd1*/+ early seeds (Figures S3A–S3G) confirmed that the aborted zygote and the arrested early endosperm were indeed the products from fertilized egg and central cells, and not from an autonomous development (Raissig et al., 2011). Further observations revealed that the aberrant zygotes in the *gcd1*/+ early seeds were distinctly shorter than those in the wild-type seeds ($p < 0.01$) (Figures S3K–S3M). However, the arrested early seeds still expressed *pDD65::H2B_GFP* specifically in the endosperm (Figures S3H–S3J) and *DD45-GFP* specifically in the zygote (Figures S3K and S3L). Thus, the arrested zygote and endosperm retained their cell-type nature, although their further development was interrupted.

Two Polar Nuclei Can Successively Fuse with a Sperm Nucleus during *gcd1* Central Cell Fertilization

To understand the behavior of the two unfused polar nuclei during fertilization in *gcd1*/+ plants, we first examined the size of the endosperm nuclei in *gcd1*/+ and wild-type early seeds and determined that they were comparable in size, with lengths of 5.65 μm (n = 27) and 5.42 μm (n = 31), respectively. Furthermore, the *gcd1*/+ and wild-type endosperm nuclei exhibited a similar variation in size, with CV values of 11% and 13.7%, respectively, indicating that endosperm nuclei in young *gcd1*/+ seeds are of uniform size and do not represent unfused polar nuclei.

We next observed aborted early seeds containing two endosperm nuclei labeled with a paternally derived AGL80-GFP signal (Figure 4J). After whole-mount clearing, each of the 17 aborted seeds contained two endosperm nuclei (Figure 4K), suggesting that two polar nuclei had fused with sperm cell. We also measured the DNA contents of the endosperm nuclei in both *gcd1*/+ and wild-type seeds at 2 DAP. The DNA contents of the endosperm nuclei ranged from 3C to 6C (Figure 4L; Table S3), confirming that each sperm cell had fused with two polar nuclei.

To trace the fusion of male and female gametes during fertilization in *gcd1* gametophytes, we used our transgenic line consisting of *pDD65::H2B_EGFP* in the *gcd1*/+ background; in this line, the central cell is labeled with GFP. After the transgenic plants were crossed with wild-type pollen carrying HTR10-monomeric red fluorescent protein 1 (Ingouff et al., 2007), unfused polar nuclei were observed in the *gcd1* gametophytes. Then, one of the polar nuclei fused with a sperm nucleus (Figures 4P–4R), followed by the other (Figures 4S–4U). Thus, in contrast to the secondary nucleus fertilization observed in *GCD1* gametophytes (Figures 4M–4O), polar nuclei in *gcd1* plants successively fuse with the sperm nucleus during central cell fertilization.

Female Gametic Cell Maturation Is Essential for Embryogenesis Initiation and Early Endosperm Development

After fertilization with wild-type pollen, the *gcd1*/+ early seeds aborted during zygote development, indicating a maternal defect in the *gcd1*/+ seeds. There are two possible mechanisms for such maternal defects. First, paternally derived *GCD1* gene expression might be repressed, or, second, the defective female gametes might lack a gametic factor necessary for postfertilization development. We investigated the first possibility by pollinating wild-type pistils with *pGCD1::H2B_GFP* pollen. The paternally derived *pGCD1::H2B_GFP* was expressed in the zygote and early endosperm immediately after fertilization (Figures 5A–5D), eliminating the possibility of epigenetic silencing of the paternal *GCD1* allele. We also pollinated wild-type pistils with *pGCD1::GCD1_GFP* pollen and found that the paternal GCD1 was expressed in zygotes and early endosperm (Figures 5E–5G). Furthermore, when *gcd1* female gametophytes were fertilized with *pGCD1::GCD1_GFP* pollen, the paternally derived GCD1 was expressed in the arrested zygote and early endosperm, but the aborted seeds could not be rescued (Figure 5H), indicating that paternal *GCD1* cues cannot rescue abnormal embryo and endosperm development.

However, in the next generation of this crossing line, we were able to detect GCD1-GFP expression in the *gcd1*/+ female gametes. When *pGCD1::GCD1_GFP- gcd1* female gametophytes were fertilized with wild-type pollen, the number of fertile ovules increased significantly (Table S2), suggesting that the aborted seeds could be rescued by *GCD1* expressed in the female gametes before fertilization but not by *GCD1* expressed in the zygote and early endosperm immediately after fertilization (Figures 5I and 5J). Thus, maturity of female gametic cells is essential for triggering embryogenesis and early endosperm development.

Central Cell–Egg Cell Communication Is Required for Their Reciprocal Development

As described above, final maturation of both central and egg cells is impaired in *gcd1* FG, blocking subsequent embryogenesis initiation and early endosperm proliferation. To explore whether the development of a central cell influences the development of its partner egg cell, and vice versa, we examined whether specific expression of the GCD1 protein in an egg or central cell in the *gcd1*/+ mutant background could functionally rescue the defective partner cell. For cell-type-specific

(J) Expression of paternally derived AGL80-GFP in a *gcd1* early seeds.
(K) Whole-mount clearing of the same seed in (J). The arrow and arrowhead indicate the same endosperm nuclei in the seed, respectively.
(L) DNA contents of endosperm nuclei in *gcd1*/+ early seeds (1–8) and wild-type (WT) ovules pollinated with wild-type pollen at 2 DAP. See also Table S3.
(M–U) Pistils of a *gcd1*/+ plant expressing *pDD65::H2B_EGFP* were pollinated with wild-type pollen carrying HTR10-mRFP1. Ovules were observed by CLSM at 8–14 HAP. (M–O) Images of a fertilized *GCD1*/+ ovule. (P–U) Images of *gcd1*/+ ovules. (M, P, and S) *pDD65::H2B_EGFP*; (N, Q, and T) HTR10-mRFP1; (O, R, and U) merged images. The dashed lines outline the embryo sacs. CCN, central cell nuclei; PN, polar nuclei; SPN, sperm cell nuclei; FPN, fused polar nuclei. Scale bars, 10 μm.
See also Figure S3 and Table S3.

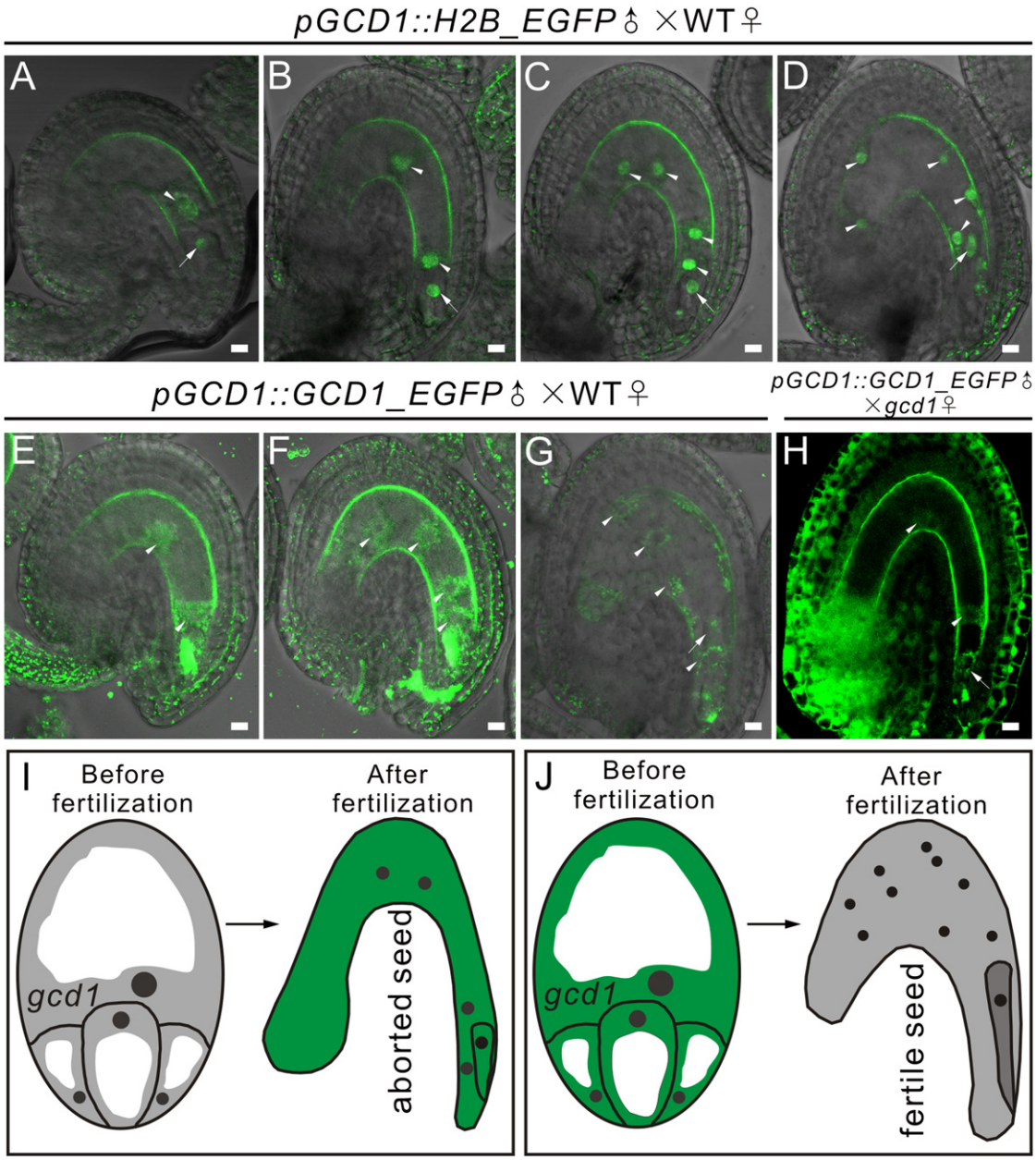


Figure 5. Paternally Derived *GCD1* Gene Expression Cannot Rescue the Arrested Zygote and Early Endosperm Development

(A–D) Paternal expression of *pGCD1::H2B_EGFP* in the zygote and early endosperm after the wild-type pistils pollinated with *pGCD1::H2B_EGFP* pollen. The arrowheads and arrows indicate the endosperm nuclei and zygote nuclei with GFP signal, respectively. (A) Fertilized egg cell and central cell. (B) Primary endosperm nucleus divided once. (C) Four endosperm nucleus stage. (D) Eight endosperm nucleus stage.

(E–H) Paternal expression of GCD1-EGFP in the zygote and early endosperm after the wild-type (E–G) or *gcd1* (H) female gametophyte pollinated with *pGCD1::GCD1_ EGFP* pollen. (E) Primary endosperm nucleus divided once. (F) Four endosperm nucleus stage. (G) Eight endosperm nucleus stage. (H) Arrested zygote and early endosperm in the *gcd1*/+ early seeds. The arrowheads indicate endosperm cytoplasmic domain with GFP signal. The arrows indicate zygote with GFP signal.

(I and J) Sketches to interpret that GCD1 expression before fertilization (J) but not after fertilization (I) can restore the arrested seeds. Green color indicates GCD1-EGFP expression. Scale bars, 10 μm.

expression of GCD1, *GCD1* was placed under the control of an egg-cell-specific *DD45* promoter (Steffen et al., 2007) or a central-cell-specific *FWA* promoter (Kinoshita et al., 2004). As expected, expression of *pDD45::GCD1* in the *gcd1*/+ egg cell suppressed the *gcd1* phenotype, decreasing the CV values for egg cell size and length (Figures 6A–6D) and dramatically increasing the proportion of egg cells that were of normal size from approximately 50% to 80% (Figures 6B–6D). Moreover, a comparison of the density distribution of egg cell size in the *gcd1/GCD1* line versus that in rescued lines confirmed this result

(Figures S4A–S4D). These results indicate that the egg cell defect in the *gcd1* FG was largely suppressed by *pDD45::GCD1* expression.

Unexpectedly, we noted that not only the egg cells but also the central cells exhibited improved final development in the *pDD45::GCD1*-rescued embryo sacs; the percentage of unfused polar nuclei in the central cells decreased dramatically, from 31% to 1.6%, with rescue of the egg cells (Figure 6G). This observation suggests that the normal development of an egg cell can promote fusion of the polar nuclei.

In the parallel experiment, the specific expression of *pFWA::GCD1* in central cells in the *gcd1*/+ mutant background suppressed the central cell developmental defect, as expected (Figure 6G). In addition, the egg cells in the *pFWA::GCD1*-rescued embryo sacs were more uniform than those in the *gcd1*/+ mutant embryo sacs (Figures 6B, 6E, 6F, S4B, S4E, and S4F), indicating that normalization of central cell development also promotes further maturation of egg cells. These observations suggest that central cells and egg cells send signals to one another to reciprocally promote final maturation of the cells.

We further analyzed embryo and endosperm development in *gcd1*/+ *pDD45::GCD1*/+ ovules pollinated with wild-type pollen. Three types of early seeds (types I, II, and III) were observed at 2 DAP (n = 195) (Figures 6H–6J). Type I seeds (80%) contained an early global embryo and developing endosperm similar to that of wild-type seeds (Figure 6H) and were significantly larger in rescued lines (carrying *pDD45::GCD1*) than in the *gcd1*/+ mutant ($p < 0.01$) (Figure 6K), indicating that the embryo and endosperm developed to the level of the restored gametic cells. Type II seeds (6.7%) contained a developing global embryo and degenerated endosperm (Figures 6I and 6K), suggesting that a small proportion of the egg cells, but none of the central cells, were rescued. Type III seeds (2%) contained arrested zygotes and arrested endosperm (Figures 6J and 6K), suggesting that they were not rescued.

We also found three types of early seeds (types 1, 2, and 3) in *gcd1*/+ *pFWA::GCD1*/+ ovules pollinated with wild-type pollen at 2 DAP (n = 210) (Figures 6L–6N). Type 1 seeds (71%) were significantly more prevalent in rescued lines ($p < 0.01$) (Figures 6L and 6O), and both the embryo and endosperm were well developed after the rescued gametic cells were fertilized. Type 2 seeds (12%) contained both developing endosperm and arrested zygotes (Figures 6M and 6O), indicating that the central cell had been rescued but that the egg cell had not. Type 3 seeds (7%) contained aborted zygotes and endosperm (Figures 6N and 6O), indicating incomplete recovery. These results further confirm that full maturation of the female gametes is necessary for seed development and that signals from the central cell or egg cell are required for subsequent embryo initiation and endosperm development.

To validate the cell-specific expression driven by the *DD45* and *FWA* promoter during female gametophyte development, we generated plasmid constructs in which the expression of the GCD1-GFP fusion protein was driven by the *DD45* or *FWA* promoter. As shown in Figures S4G–S4L, the egg- and central-cell-specificity of the GCD1-GFP fusion protein were detected in the respective *gcd1*/+ *pDD45::GCD1_GFP*/– and *gcd1*/+ *pFWA::GCD1_GFP*/– lines even before polar nucleus fusion but not before cellularization in embryo sacs (n > 50). Both transgenes caused a significant increase in the seed set of the *gcd1*/+ mutant (Table S2). In addition, gametic cell development and subsequent early embryogenesis and endosperm development was effectively restored. These results are in accordance with the results and conclusions described for the *pDD45::GCD1*- and *pFWA::GCD1*-transgenic *gcd1*/+lines described above.

Furthermore, we confirmed that GCD1 expression in the embryo sacs of wild-type, *gcd1*/+ *pFWA::GCD1*/+ and *gcd1*/+ *pDD45::GCD1*/+ lines was in the similar physiological level (Figure S4M–S4O).

GCD1 Localizes in the Mitochondria and Is Required for Crista Integrity in Female Gametes

To provide insight into the structural basis of the signals related to *GCD1* function, we examined the subcellular localization of the GCD1 protein using transgenic *Arabidopsis* plants expressing a GCD1-GFP fusion protein driven by the 35S promoter. The colocalization of GCD1-GFP and mitochondria (labeled with MitoTracker Red) in root cells (Figures 7A–7C) indicated that the GCD1 protein localized in mitochondria.

Ultrastructural analysis of the central cell mitochondria of *gcd1* gametophytes revealed an irregular structure and a reduced number of cristae (Figures 7D and 7E). In contrast, there were numerous, clearly visible cristae in the mitochondria in the *gcd1*/+ integument cell of the same ovule (Figure 7F) and in the wild-type embryo sac (Figure 7G), indicating that *GCD1* is required for the integrity of female gametic mitochondria.

Mitochondrial Dysfunction Blocks Maturation of the Female Gametes and Transmission of Their Developmental Signals

GCD1 functions in mitochondrial crista integrity; however, whether there is a causal relationship between the integrity of gametic mitochondria and the final maturation of the gametes is unconfirmed. To address this issue, we specifically impaired mitochondrial function in central cells and egg cells of wild-type plants via site-specific mutagenesis of ADP/ATP carrier protein 2 (AAC2) at amino acid residue 199. We expressed this dominant allele, fused to *GFP*, from a central cell promoter (Kägi et al., 2010). The *Arabidopsis aac2* mutant has been previously used for similar purpose by Kägi et al. (2010). AAC2 localizes at the inner membrane of mitochondria. Its mutations in yeast impair the electron transport chain and result in membrane uncoupling. The *pMEA::aac2*^{A199D}*_GFP* construct was expressed specifically in the central cell, as expected (Figure 8A). Analysis of the homozygous, independent transgenic lines confirmed that central cell maturation was affected in the *aac2* mutant, as evidenced by the presence of unfused polar nuclei and nondegraded antipodal cells (Figures 8B–8F). Interestingly, reduced egg cell size was also found in embryo sacs (Figures 8G–8I). The CV of the *pMEA::aac2*^{A199D}*_GFP* egg cells was much higher (by almost 50%) than that of wild-type egg cells (Figures 8J–8L), and 50.5% of the *pMEA::aac2*^{A199D}*_GFP* egg cells were of abnormal size (compare to 6.8% in the wild-type strain) (Figures 8J–8L). The two strains also differed markedly in their density distributions of egg cell size (Figures S5A–S5C), indicating a defect in *pMEA::aac2*^{A199D}*_GFP* egg cell maturation. These findings suggest that the induced mitochondrial dysfunction impeded central cell maturation, blocking the transmission

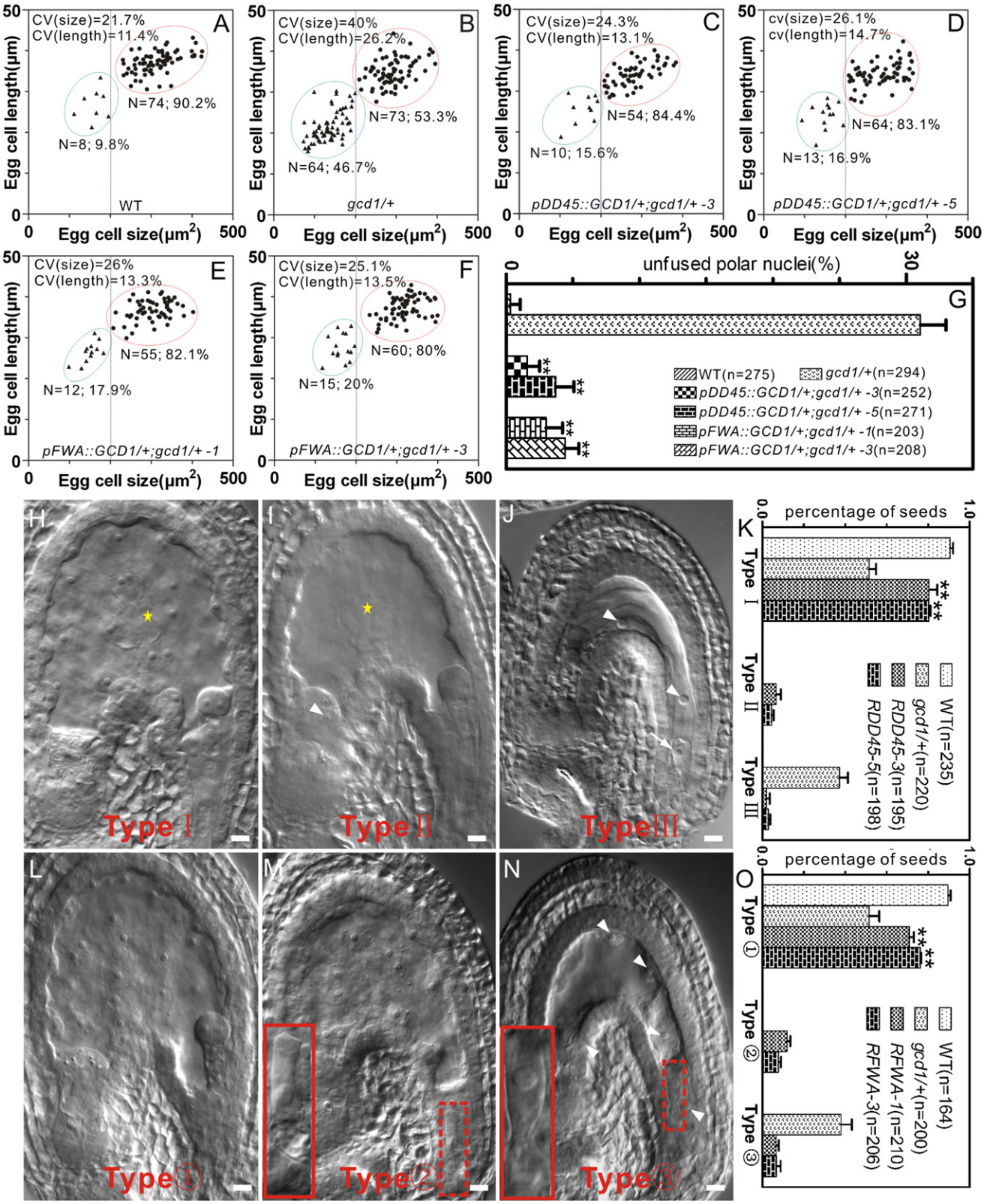


Figure 6. Reciprocal Signals between Female Gamete Cells

(A–F) The distribution of egg cell sizes and lengths in the wild-type (A), *gcd1*/+ (B), two independent transgenic lines with *pDD45::GCD1*/*pDD45::GCD1* in *gcd1*/+ background (C and D), and two independent transgenic lines with *pFWA::GCD1*/*pFWA::GCD1* in *gcd1*/+ background (E and F).

of the developmental signal from the central cells and subsequently blocking egg cell maturation.

Similarly, egg-cell-specific expression of the *aac2*^{A199D}*_GFP* construct from the *DD45* promoter significantly affected egg cell development (Figure 8M), as evidenced by increases in the proportion of egg cells of abnormal size and in the egg cell CV (Figures 8N–8P) and by an abnormal density distribution curve (Figures S5D–S5F). As expected, unfused polar nuclei were observed in the central cells (Figure 8Q). Notably, antipodal degeneration was also suppressed (Figure 8R), confirming a defect in central cell development.

Moreover, we crossed the two *aac2* transgenic lines to disrupt the signaling between female gametes. As expected, *aac2* expressed in both central cell and egg cell (Figures S5G and S5H). At the same time, compared to each of the two *aac2* transgenic lines early embryogenesis and endosperm development has been affected much more seriously (Figures S5I–S5Q).

These results provide further evidence for the existence of egg cell-central cell communication and suggest that this signaling is mitochondrion regulated.

DISCUSSION

GCD1 Is Required for Female Gametic Maturation in *Arabidopsis*

In most flowering plants, a female gametophyte originates from a single haploid spore via three sequential mitotic nuclear divisions. In *Arabidopsis*, the process is divided into seven continuous stages (FG1–FG7), with female gametes generated in the FG5 stage (Christensen et al., 1997). The central cell and egg cell then mature through cell growth, vacuole expansion, and polarity construction. Dramatic increases in the size of the egg cell are then observed. A typical characteristic of central cell maturation is the presence of one secondary nucleus with a large vacuole (Christensen et al., 1997; Huang and Russell, 1992; Russell, 1993).

Genetic analyses have revealed numerous genes that play an important role in the development of female gametophytes (Ma and Sundaresan, 2010; Yang et al., 2010). However, little is known about the mechanism of female gametic specification and maturation (Gross-Hardt et al., 2007; Johnston et al., 2008; Moll et al., 2008; Pagnussat et al., 2007, 2009; Sprunck and Gross-Hardt, 2011). The type I MADS-box genes (*DIA* and *AGL80*) have been reported to play an important role in the development of central cells. In the *diana* (*dia, agl61*) mutant, polar nuclei of the central cell are not fused, and central cells have an aberrant morphology (Bemer et al., 2008; Steffen et al., 2008). DIA forms a heterodimer with AGL80, and a mutation in the *AGL80/FEM111* gene in *Arabidopsis* specifically affects central cell maturation (Portereiko et al., 2006a). Polar nuclei fusion is also affected in *magatama3* (*maa3*) mutants. MAA3 may regulate the RNA metabolism that is responsible for nucleolar organization of the central cell (Shimizu et al., 2008).

Electron microscopy has revealed that the fusion of the polar nuclei begins with their contact with endoplasmic reticulum membranes that are continuous with the outer nuclear membranes of the polar nuclei (Jensen, 1964). The 70 kDa heat shock protein known as binding immunoglobulin protein (BiP) acts as a molecular chaperone in the endoplasmic reticulum, and the *bip1bip2* double mutation also affects polar nuclei fusion (Maruyama et al., 2010). Interestingly, most mutations blocking polar nuclear fusion are in genes encoding proteins that locate in mitochondria, including *NUCLEAR FUSION DEFECTIVE1* (*NFD1*), *NFD3*, *NFD4*, *NFD5*, *NFD6* (Portereiko et al., 2006b), *GFA2* (Christensen et al., 2002), and *SYCO1* (Kägi et al., 2010).

Our findings indicate that GCD1, a mitochondrial protein, plays an important role in both central cell and egg cell maturation via its effect on the integrity of mitochondria cristae. Interestingly, none of the early developmental processes occurring during embryo sac development, including nuclear division, migration, cellularization, and cell fate determination, are affected by the *gcd1* mutation, indicating that GCD1 functions critically in the process of female gametic maturation.

Maturation of the Female Gamete Is Essential for Initiation of Embryogenesis and Early Endosperm Proliferation

Fusion of the male and female gametes, leading to zygotic activation and embryogenesis initiation, is one of the most decisive events in sexual reproduction (Stitzel and Seydoux, 2007). During double fertilization in higher plants, the two female gametes fuse with sperms, resulting in the embryo and endosperm. Our data show that *gcd1* female gametes can fuse with wild-type sperm cells, but zygote and early endosperm development is arrested, indicating blockage of some key process required for embryogenesis initiation and early endosperm proliferation. Thus, the female gametic maturation process is essential in seed setting. Without the maternal molecular background established during female gametic maturation, the paternal genome by itself cannot initiate embryogenesis or ensure endosperm development after fertilization.

(G) Both the *pDD45::GCD1* and *pFWA::GCD1* constructs restored the polar nuclei fusion defects in *gcd1*/+ plants. Gametophytes were analyzed 2 days after emasculation.

(H–J) Embryo and endosperm development after *gcd1/GCD pDD45::GCD1*/+ pistils were pollinated with wild-type pollen. Three types of seeds were observed at 2 DAP. (H) Type I seed. (I) Type II seed. (J) Type III seed. Asterisks indicate the expanded embryo sac; arrows indicate the arrested zygote; arrowheads indicate the aborted endosperm nuclei.

(K) The percentage of different types of early seeds in the wild-type, *gcd1*/+, and two homozygous rescue lines in the *gcd1*/+ background by *pDD45::GCD1* (*RDD45-3* and *RDD45-5*).

(L–N) Embryo and endosperm development after *gcd1*/+ *pFWA::GCD1*/+ pistils were pollinated with wild-type pollen. Three types of seeds were observed at 2 DAP. (L) Type 1 seed, (M) type 2 seed, and (N) type 3 seed. Insets in (M) and (N) are enlarged images of the framed areas (dash line); arrowheads indicate the aborted endosperm nuclei.

(O) The percentage of different types of early seeds in the wild-type, *gcd1*/+, and two homozygous rescue lines in the *gcd1*/+ background by *pFWA::GCD1* (*RFWA-1* and *RFWA-3*). $^{**}p < 0.01$. Scale bars, 10 μm. Error bars indicate SD.

See also Figure S4.

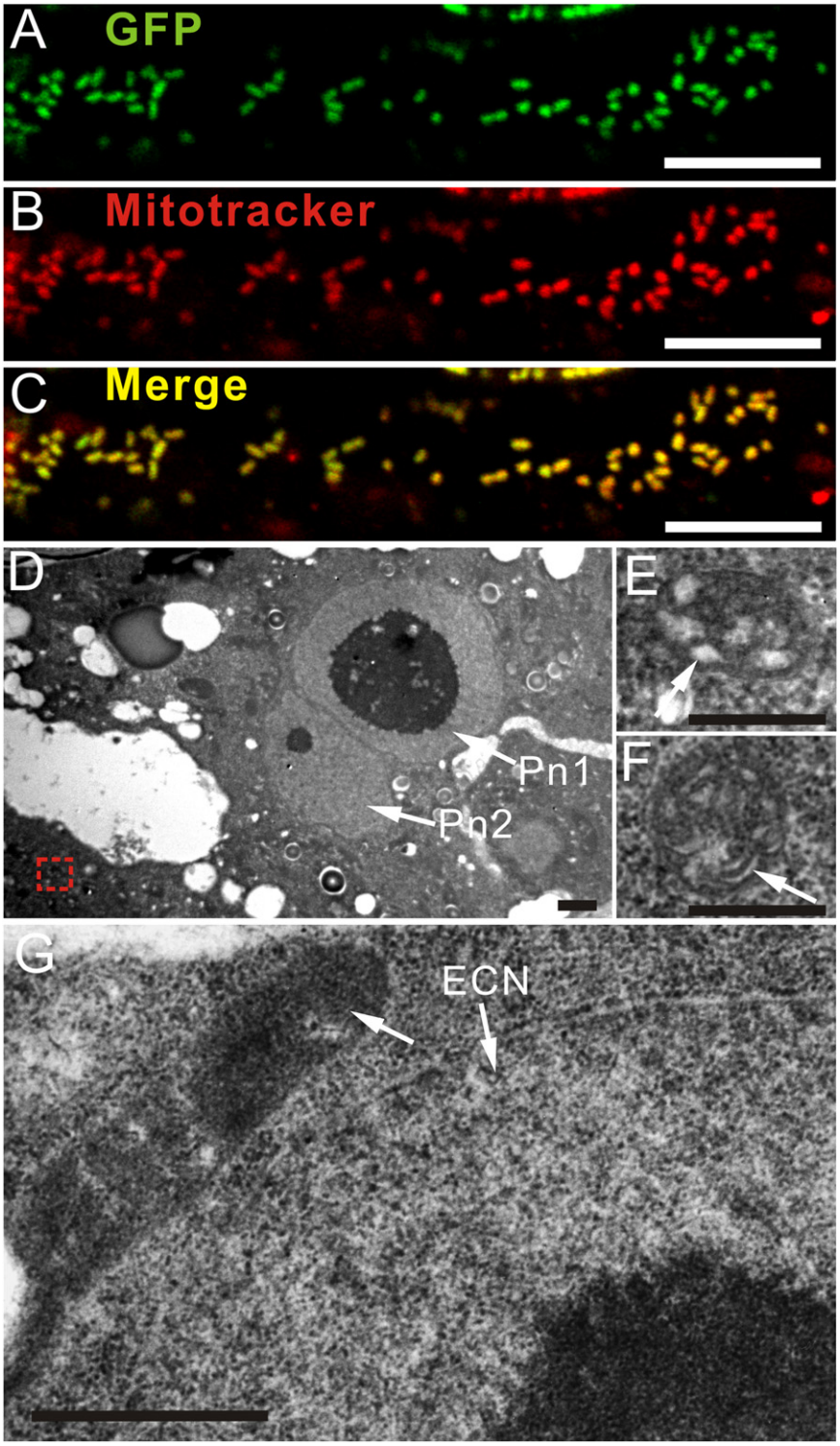


Figure 7. GCD1 Localizes in Mitochondria and Is Necessary for Cristae Integrity in the Central Cell

(A–C) Subcellular localization of GCD1-GFP in root cell. (A) *p35S::GCD1_GFP*. (B) MitoTracker red. (C) Merged image. Scale bars, 10 μm.

(D) Ultrastructural analysis revealed unfused polar nuclei (arrows indicate) of *gcd1* female gametophyte 12 hr after emasculation. Pn, polar nuclei. Scale bar, 1 μm.

(E) Magnification of boxes in (D): central cell mitochondria. Arrows indicate cristae. Scale bar, 500 nm.

(F) Integument cell mitochondria of the same ovule in (D). Arrows indicate cristae. Scale bar, 500 nm.

(G) Wild-type mitochondria in the FG. ECN, egg cell nucleus. Arrows indicate cristae. Scale bar, 1 μm.

In animals, embryogenesis initiation relies primarily on maternally deposited mRNAs and proteins stored in the oocyte prior to fertilization; the young embryo is transcriptionally quiescent (Baroux et al., 2008; Schier, 2007; Tadros and Lipshitz, 2009). The extent of maternal influence on embryogenesis initiation in angiosperms is less clear. Few genes have been shown to be related to embryogenesis initiation. The *FAC1* gene, which encodes an adenosine monophosphate (AMP) deaminase, is essential for zygotic division, possibly acting through AMP depletion to provide sufficient energy for the zygote to proceed through development (Xu et al., 2005). The *ZEUS1* gene, which encodes a thymidylate kinase that regulates the G1/S-phase transition, is essential for promoting the first division of the zygote (Ronceret et al., 2008). However, whether these protein factors are maternally deposited in the egg cell is unknown. Recent studies have identified de novo expressed transcripts in zygotes or two-celled proembryos (Meyer and Scholten, 2007; Ning et al., 2006; Sprunck et al., 2005; Zhao et al., 2011), and another study has confirmed that endosperm development depends on de novo transcription (Pillot et al., 2010). These data indicate that de novo transcripts play an essential role in zygote development or early embryogenesis in higher plants.

Although we observed the successful fertilization of *gcd1* female gametes by wild-type sperm cells, indicating that the maternally deposited protein is sufficient for this process, we also observed that the fertilized *gcd1* female gametes failed to develop seeds. This latter observation suggests that fertilized *gcd1* female gametes may suffer from insufficient de novo transcription for successful embryogenesis and endosperm development. If the necessary molecular machinery for genome activation and de novo transcription must be established during gametic maturation, then blocking the maturation process would also arrest the activation process. Although this hypothesis remains to be tested, it is clear that the final

maturation of both gametes is essential for triggering embryogenesis and endosperm development.

Egg Cell–Central Cell Communication Ensures Coordinated Development of Female Gametes

We found that egg cell development affects central cell development and vice versa. Specific rescue of a *gcd1* egg cell also rescued its partner central cell, and specific rescue of a *gcd1* central cell partially rescued its partner egg cell, indicating that the gametes emit reciprocal signals that provide for their coordinated development. Specific disruption of the development of either egg cells or central cells in wild-type plants, by interrupting their mitochondrial function, partially affected the maturation of both types of female gametic cells, confirming that these developmental signals are necessary for final gametic maturation and are mitochondrion regulated. However, because of technical limitation we cannot completely exclude the possibility that the promoters we used have a residual expression that is not easily detectable in neighbor cells when egg or central-cell-specific rescue and mitochondrion malfunction experiments were performed. To assess the possibility and the role of these few molecules in egg or central cell development, we have also used promoter *DD31* to drive *GCD1* expression in *gcd1/GCD1* background, which confers *GCD1* specially expression in synergid cells. But, none of the transgenic lines showed restoration of *gcd1/GCD1* female gamete development and seed fertility (Table S2). This suggests that even if the residual expression exists it may not play a notable role alone in female gamete maturation. It is possible that the few molecules combine with the signals from neighbor gamete cell together to contribute to the central cell and egg cell final maturation. We also crossed the two *aac2* transgenic lines and found early embryogenesis and endosperm development were affected much more seriously, compared to each of the two *aac2* transgenic lines. These results support the existence of egg cell-central cell communication and imply that this signaling plays a critical role in gamete development. Interestingly, a recently published report demonstrated *LACHESIS*-dependent regulation of egg cell signaling in developing female gametophytes and suggested that cell in the female gametophyte is orchestrated by the egg cell (Völz et al., 2012). Also, a recent analysis on a secreted peptide ZmEAL1 confirmed the involvement of egg cell signaling in controlling antipodal cell fate in maize (Krohn et al., 2012). Integrating all these data will clarify the roles and mechanisms of cell-to-cell communication among cells in the female gametophyte.

Unlike fertilization in animals, double fertilization in angiosperm plants requires fusion of both the central cell and egg cell with sperm cells, thus requiring the coordinated development of the female gametes for successful synchronous fertilization. However, how the synchronous development of these gametes is regulated has been largely unclear. The cell-to-cell communication between female gametes revealed in the present work ensures that the central cell and egg cell develop correctly and synchronously, thereby enabling effective double fertilization. Based on these findings, we propose that the maturation of female gametes is controlled by two signaling pathways: autonomous regulation via control of mitochondrial function and mitochondrion-regulated reciprocal signaling between the central cell and egg cell for the coordinated development of female gametes. These data may explain the developmental relationship between female gametes.

EXPERIMENTAL PROCEDURES

Plant Material and Growth Conditions

A. thaliana qrt1 (Preuss et al., 1994) was used as a wild-type strain. The *gcd1-1* allele was isolated from our mutant library; the *gcd1-2* (*Sail_58_C10*) allele was obtained from the ABRC. Other materials and culture conditions are described in the Supplemental Experimental Procedures.

Mutant Library Construction and Genotype Identification

We generated an *Arabidopsis* mutant library with T-DNA encoding *LAT52::EGFP*, a cell-autonomous pollen-specific reporter (Sessions et al., 2002; Twell et al., 1989), and a hygromycin-resistance gene. T-DNA mutagenesis was carried out on *qrt1* plants, which maintains male meiotic products in tetrads (Preuss et al., 1994). The mutant genotype was identified by PCR and by the number of microspore with GFP fluorescence. A detailed description is provided in the Supplemental Experimental Procedures.

Egg Cell Size Calculation

Confocal laser scanning microscope (CLSM) analysis of ovules was performed as described by Christensen et al. (1997). To analyze female gametophytes at the terminal developmental stage (stage FG7), we emasculated flowers at stage 12c, waited 24 hr and harvested the pistils for fixation. And, single image section with the most egg cell sectional area was captured using a FV1000 CLSM (Olympus). The size and length of egg cells were calculated using Image J software.

CLSM and Histological Analysis

The fresh ovules were excised from siliques and mounted on a slide in 80 mM sorbitol for CLSM analysis. Mitochondria were stained by incubating seedlings in 100 nM MitoTracker Red CMXRos (Invitrogen, Carlsbad, CA, USA) in the dark for 30 min before imaging. The DNA content of nuclei was examined in optical sections of the seeds stained with propidium iodide. The average DNA content of 15 diploid sporophytic nuclei in the integument cells of the same seed was used as references for the diploid DNA content. A detailed explanation is provided in the Supplemental Experimental Procedures.

ACCESSION NUMBERS

The GenBank accession number for the GCD1 protein sequence is AED97589.1. The *Arabidopsis* Genome Initiative number for the protein sequence of GCD1 is At5g62270.1.

SUPPLEMENTAL INFORMATION

Supplemental information includes five figures, three tables, and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.devcel.2012.09.011.

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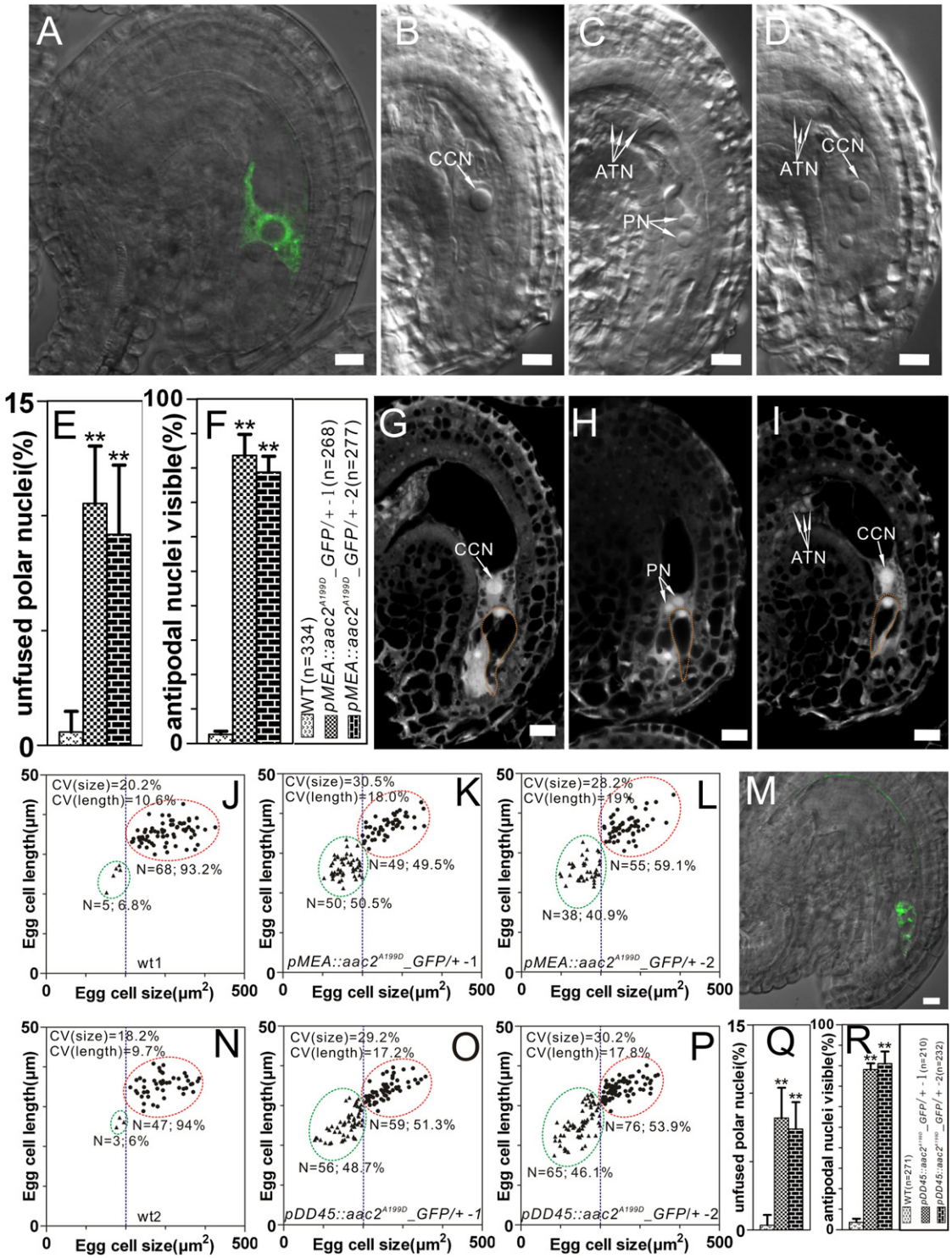

Figure 8. The Expression of *aac2*^{A199D} in the Central Cell or Egg Cell of Wild-Type Plants, Respectively, Caused Unfused Polar Nuclei and Immature Egg Cell

(A) Localization of *pMEA::aac2*^{A199D}*_GFP* in the central cell.

(B–D) Whole-mount clearing of wild-type (B) and *pMEA::aac2*^{A199D}*_GFP* (C and D) ovules 2 days after emasculation.

(E and F) Frequencies of unfused polar nuclei (E) and persistent antipodal cells (F) in wild-type and two independent homozygous *pMEA::aac2*^{A199D}*_GFP* transgenic lines. Gametophytes were analyzed 2 days after emasculation.

(G–I) CLSM analysis of wild-type (G) and *pMEA::aac2*^{A199D}*_GFP* (H and I) female gametophyte. The dashed lines outline the egg cells.

(J–L) The distribution of egg cell sizes and lengths in the wild-type (J) and two independent homozygous *pMEA::aac2*^{A199D}*::GFP* transgenic lines (K and L).

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(M) Localization of *pDD45::aac2^{A199D}_GFP* in the egg cell of the female gametophyte.
(N–P) The distribution of egg cell sizes and lengths in the wild-type (N) and two independent homozygous *pDD45::aac2^{A199D}_GFP* transgenic lines (O and P).
(Q and R) Frequencies of unfused polar nuclei (Q) and persistent antipodal cells (R) in wild-type and two independent homozygous *pDD45::aac2^{A199D}_GFP* transgenic lines. Gametophytes were analyzed 2 days after emasculation. CCN, central cell nuclei; PN, polar nuclei; ATN, antipodal cell nuclei. $^{**}p < 0.01$. Scale bars, 10 μm. Error bars indicate SD.
See also Figure S5.

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